DE GRUYTER Pure Appl. Chem. 2019; 91(3): 489–496

Conference paper

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Formation of highly quaternized *N*,*N*,*N*-trimethylchitosan: a chemoselective methodology in aqueous media

https://doi.org/10.1515/pac-2018-0924

Abstract: *N*,*N*,*N*-Trimethylchitosan (TMC) represents a rare example of cationic polysaccharides and numerous studies have shown its potential in biological and biomedical applications. TMC with high degrees of quaternization (DQ) were synthesized from *N*-methylation of *N*,*N*-dimethylchitosan (DMC), which was obtained by reductive alkylation of high molecular weight chitosan in a simple step process and in good yields. The effects of base and solvents were evaluated on the quaternization reaction. The *N*-methylation of DMC was performed selectively by CH_3I and carbonate in water where quaternization was achieved quantitatively with a low degree of *O*-methylation (17 %). Moreover, the greener procedure allows easy recovery and purification by conventional filtration as a carbonate salt, in which the anion can be exchanged by an acid-base reaction. Quantification of DQ involving ^1H NMR integration of methyl peaks must be performed on protonated TMC. High field NMR spectra of TMC showed two specific chemical shifts for anomeric peaks (5.0 and 5.4 ppm) that can also be used for the determination of DQ. This latter method avoids the superimposition problems with other pyranosyl peaks.

Keywords: cationic polymer; degree of quaternization; *N*-alkylation; *N*,*N*,*N*-trimethylchitosan; POC-17; quantification.

Introduction

Chitosan or poly[ß-(1→4)-2-amino-2-deoxy-D-glucopyranose] is the second most abundant biopolymer on Earth after cellulose. Chitosan is a biocompatible, biodegradable and non-toxic linear polysaccharide obtained mainly from *N*-deacetylation of chitin, which is obtained commercially from crustacean shells. However, the solubility of chitosan in water is limited at physiological pH (>6.0) [1]. The formation of *N*,*N*,*N*-trimethylchitosan (TMC) from chitosan allows the introduction of positive charges and the achievement of a cationic water-soluble polymer, that does not depend on pH. TMC was studied in numerous applications such as drug [2–4] and gene delivery [5, 6], antibacterian [7, 8], wound healing [9], and new materials [10, 11]. The density of positive charge is known to enhance the drug absorption by TMC [7]. TMC is known to open the tight junction of epithelial cells and has been proven to be a potent intestinal absorption enhancer for hydrophilic and macromolecular drugs in physiological pH [12–14]. A structure activity relationship study reveals that *N*-quaternization on chitosan was responsible of the antibacterial activity against *Staphylococcus aureus* [15].

Article note: A collection of papers presented at the 17th Polymers and Organic Chemistry (POC-17) conference held 4–7 June 2018 in Le Corum, Montpelier, France.

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High degree of quaternization (DQ) and chemoselectivity represent therefore an important goal for the formation of TMC. The synthesis of TMC has already been reported by successive methylation reactions of chitosan with methyl iodide and a base, such as sodium hydroxide, under various experimental conditions using organic solvents [16, 17]. However, alkylation of chitosan in these conditions leads to a mixture of unmethylated, mono, di and trimethylated amines. A high DQ on chitosan can be obtained (DQ of 90.5 %) in three methylation steps using iodomethane and sodium hydroxide, but these conditions also lead to high degrees of *O*-3 and *O*-6 methylation corresponding to a degree of substitution of 82.8 and 98.5 %, respectively [18]. The formation of methyl ether (*O*-methylation) by substitution of alcohol group decreases the solubility of chitosan derivatives and could lead to a water insoluble product [16, 18, 19].

Experimental conditions were studied to prevent *O*-methylation and generally lead to low DQ [18]. The utilization of sodium hydroxide or strong alkaline environment could decrease the molecular weight of the polymer by hydrolysis. *N*,*N*-dimethylaminopyridine was used as an alternative base to sodium hydroxide for avoid degradation of chitosan but low DQ (7.3–9.6 %) was obtained [17]. TMC having a DQ of 61 % was also synthesized by *N*-methylation of *N*,*N*-dimethylchitosan (DMC) in *N*-methyl-2-pyrrolidone without addition of base followed by an anion exchange [20, 21].

In this work, we report a mild, efficient and chemoselective procedure for the formation of TMC possessing high DQ by *N*-methylation of DMC in water. The TMC was obtained from high molecular weight chitosan. Moreover, the quantification of the DQ on TMC using the integration of anomeric signals is better than integration of methyl peaks.

Experimental

General information

High molecular weight chitosan from Nordic shrimp (*Pandalus borealis*) shells possessing a degree of deacetylation of 95.9 % and a viscosity in a 1 % acetic acid aqueous solution of 157 cps was purchased from Marinard Biotech Inc. (Gaspé, Canada). Chitosan was mechanically ground to 0.6 mm before utilization. Deionized water was obtained using a Nanopure Diamond system (model D11931) from Barnstead. Ultrafiltrations were realized with regenerated cellulose filtration membranes (Amicon YM) possessing a cut-off of 30 000 NMWL in a stirred cell bought from Millipore. IR and NMR spectra were recorded respectively from a PerkinElmer model 1600 series IR spectrometer using KBr pellets and Bruker 600 MHz NMR spectrometer. Coupling constants are given in Hertz. DMU and TMU is the abbreviation of *N*,*N*-dimethylated units and *N*,*N*,*N*-trimethylated units, respectively. Elemental analyses of chitosan derivatives were performed on a Costech 410 elemental analyzer.

Determination of degree of substitution by NMR spectroscopy

The DQ were determined from the integral ratio between trimethyl and pyranosyl hydrogens. The degrees of substitution in percentage were determined using the following equations from ^{1}H NMR spectra of protonated TMC with HCl in D_2O:

$$\text{Degree of quaternization (\%)} = ([N(CH_3)_3]\times 6)/([\text{H-2, H-3, H-4, H-5, H-6, H-6'}]\times 9)\times 100$$

$$\text{Degree of dimethylation (\%)} = [N(CH_3)_2]/[\text{H-2, H-3, H-4, H-5, H-6, H-6'}]\times 100$$

$$\text{Degree of } N\text{-acetylation (\%)} = ([C{=}O(CH_3)]\times 6)/([\text{H-2, H-3, H-4, H-5, H-6, H-6'}]\times 3)\times 100$$

$$\text{Degree of } O\text{-methylation (\%)} = ([O(CH_3)\times 6])/([\text{H-2, H-3, H-4, H-5, H-6, H-6'}]\times 3)\times 100$$

where [$N(CH_3)_3$], [$N(CH_3)_2$], and ([$C=O(CH_3)$] are the integrals of the *N*,*N*,*N*-trimethyl (δ 3.30 ppm), *N*,*N*-dimethyl (δ 3.00 ppm), and acetyl (δ 2.0 ppm). [$O(CH_3)$] represents the integrals of methyl peaks at 3.35 and 3.43 ppm for the degrees of 3-*O*- and 6-*O*-methylation, respectively. [H-2, H-3, H-4, H-5, H-6, H-6′] corresponds to the integrals of 6 pyranosyl protons at 3.6–4.6 ppm, excluding the anomeric hydrogens (δ 5.0–5.5 ppm).

On the other hand, the DQ can also be determined from the integrals of anomeric protons from this equation.

$$\text{Degree of quaternization (\%)} = ([\text{H-1 TMU}]/([\text{H-1 TMU}]+[\text{H-1 DMU, H-1 GluNAc}]))\times 100$$

where [H-1 TMU] and [H-1 DMU, H-1 GluNAc] are the integrals of anomeric protons of TMU at 5.4 ppm and of the superimposition of DMU and *N*-acetyl-2-amino-2-deoxyglucopyranosyl units at 5.0 ppm.

Formation of *N*,*N*-dimethylchitosan

Chitosan (12 g, 73.7 mmol of glucopyranosyl units) was dissolved in 600 mL of an aqueous solution containing 28 mL of 88 % formic acid. Formaldehyde (21 mL, 37 %) was added to the reaction mixture that was stirred and heated to 70 °C. After 12 h, the solution was allowed to reach the room temperature and was treated with sodium hydroxide 5N (~300 mL) to reach pH 11–12. The resulting suspension was filtered and washed with water until the filtrate was pH 7. The solid was then washed with ethanol (25 mL) followed by diethyl ether (25 mL). Finally, the solid was dried at normal atmosphere. An off-white solid was obtained with a yield of 73 % (10.21 g). IR υ (cm^{-1}) 3449 (OH), 2927 and 2881 (CH), 1658 (C=O), 1481 (CH_3 def), 1000-1200 (C-O), 851. ^1H NMR in D_2O of DMC protonated with HCl, δ 5.03 (d, 1H, $^1J_{1,2}$ = 6.7, H-1), 4.17 (t, 1H, J = 9.1, H-3), 4.03 (t, 1H, J = 8.6, H-4), 3.87 (broad, 1H, H-5), 3.79 (d, 1H, J = 7.6, H-6), 3.69 (d, 1H, J = 8.8, H-6), 3.32 (t, J = 8.7, H-2), 2.99 (s, 6H, CH_3), 1.98 (AcN) ppm. ^{13}C NMR in D_2O of DMC protonated with HCl, δ 95.05 (C-1), 75.50 (C-4), 74.53 (C-5), 68.16 (C-3), 67.31 (C-6), 60.35 (C-2), 41.88 (CH_3) ppm.

Formation of TMC iodide with a DQ of 48 %

A suspension of DMC (0.400 g, 2.11 mmol of glucopyranosyl units), sodium bicarbonate (0.532 g, 6.33 mmol) and sodium iodide (0.825 g, 5.50 mmol) in 100 mL DMF/water (90/10 v/v) was treated with iodomethane (0.80 mL, 12.8 mmol) and heated at 75 °C during 8 h. Successive additions of iodomethane (0.80 mL) and sodium bicarbonate (0.532 g, 6.33 mmol) was realized after 2, 4 and 6 h. The solution was evaporated, and the resulting solid was dissolved in water (75 mL). The solution was ultrafiltered to a volume of 10 mL and washed twice with water (65 mL). The polymeric solution was concentrated (2–3 mL) by evaporation before to be precipitated with a mixture of acetone and diethyl ether (65 mL). The solid was filtered, washed with diethyl ether (50 mL) and dried overnight under vacuum. An off-white solid was obtained with a yield of 100 % (0.442 g). IR υ (cm^{-1}) 3385 (OH), 2936 and 2879 (CH), 1654 (C=O), 1473, 900-1200 (C-O). ^1H NMR in D_2O, δ 5.44 (br s, 1H, H-1 TMU), 5.05 (d, 1H, $^1J_{1,2}$ = 6, H-1 DMU), 4.42 (t, 1H, J = 5, H-3 TMU), 4.31 (t, 1H, J = 7, H-4 TMU), 4.20 (t, 1H, J = 9, H-3 DMU), 4.07 (t, 1H, J = 8, H-4 DMU), 3.92 (br s, 2H, H-6 DMU TMU), 3.86 (d, 1H, J = 11, H-6 TMU), 3.80 (br s, 2H, H-5 DMU TMU), 3.73 (d, 1H, J = 9, H-6 DMU), 3.69 (t, J = 4, H-2 TMU), 3.50 (s, O-CH_3), 3.39 (s, O-CH_3), 3.34 (t, J = 8.5, H-2 DMU), 3.29 (s, 9H, $N(CH_3)_3$), 2.99 (s, 6H, $N(CH_3)_2$), 2.02 (br s, NAc).

Formation of TMC chloride with a DQ of 95 %

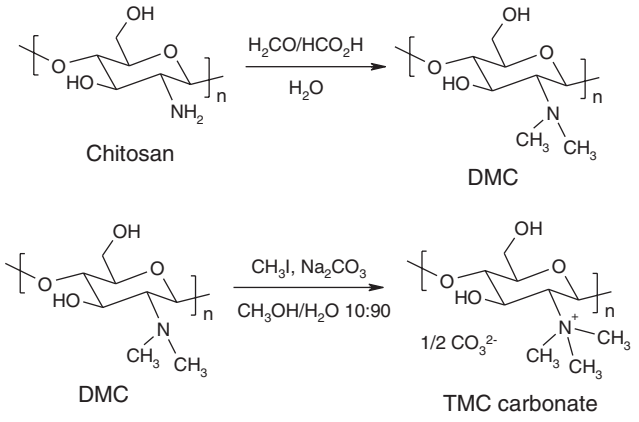
A suspension of DMC (0.600 g, 3.17 mmol of glucopyranosyl units) and sodium iodide (1.23 g, 8.20 mmol) in 100 mL of water/methanol (90/10 v/v) was treated with iodomethane (1.20 mL, 19.3 mmol) and sodium carbonate (1.01 g, 9.51 mmol). This mixture was heated at 70 °C during 46 h. Successive additions of iodomethane

(1.20 mL, 19.3 mmol) were carried out after 2, 4, 6, 22, 24, 26 and 28 h. The suspension was filtered and the solid was washed with water (50 mL). The resulting solid was suspended in water (90 mL), acidified with HCl (1 M) during 30 min., brought to pH 8–9 with NaOH (10 M), ultrafiltered (3 × 75 mL), concentrated by evaporation and finally precipitated with ethanol (75 mL). The obtained solid was filtered, washed with diethyl ether (50 mL) and dried overnight under vacuum. A light yellow solid was obtained (0.442 g) in 90 % yield. IR υ (cm^{-1}) 3385 (OH), 2936 and 2879 (CH), 1654 (C=O), 1473, 900-1200 (C-O). ^{1}H NMR in D_2O, δ 5.43 (d, 1H, $^1J_{1,2}$ = 2.8, H-1), 4.40 (t, 1H, J = 5, H-3), 4.30 (t, 1H, J = 7–8, H-4), 3.93 (d, 1H, H-6), 3.86 (br, 1H, H-5), 3.79 (d, 1H, H-6), 3.67 (t, J = 3.8, H-2), 3.29 (s, 9H, CH_3), 2.03 (AcN) ppm. ^{13}C NMR in D_2O, δ 165.22 (C=O Ac), 96.64 (C-1), 78.82 (C-4), 77.20 (C-5), 75.79 (C-3), 68.22 (C-6), 61.23 (C-2), 54.13 (Me), 20.70 (Ac) ppm. Anal. Calcd for $C_{8.96}H_{17.79}NO_{4.07}Cl_{0.96}$: C, 40.32; N, 5.28. Found: C, 40.39; N, 5.33.

Results and discussion

Formation and characterization of *N*,*N*-dimethylchitosan (DMC)

The selectivity of alkylation as well as a high DQ are very important for the properties of TMC including the water solubility. Direct alkylation of chitosan with iodomethane and a base leads to the formation of a mixture of mono, di and tri- *N*-methylated chitosan as well as *O*-methylation. The Eschweiler-Clarke methylation has the advantage to be realized in aqueous acidic medium in which the chitosan is soluble and where no alkylation of alcohol groups is achieved. However, the Eschweiler-Clarke reaction stops to tertiary amine and a subsequent *N*-alkylation reaction must be realized to attain TMC. In our work, DMC is obtained rapidly with a good yield and a high degree of substitution (DS) by reaction of high molecular weight chitosan with formaldehyde in a formic acid solution (Scheme 1). DMC is soluble in acidic aqueous solution and insoluble in DMSO, ethanol and water at pH 7. ^{1}H NMR spectrum shows a singlet at 3.0 ppm corresponding to an integration of six protons which indicates the occurrence of *N*-methyl groups with an excellent DS of 2.0. The methyl groups were also observed in ^{13}C NMR at 41.9 ppm. The reaction completeness can be explained by the *in situ* reduction of the imine by the formate ion that drives the equilibrium toward the products. In the pyranosyl region (3.3–5.3 ppm) of DMC, seven peaks corresponding to one hydrogen atom each, are observed on the ^{1}H NMR spectrum as shown in Fig. 1. The homonuclear proton coupling is observed that is consistent with the glucopyranosyl structure. IR spectrum of DMC (Fig. 2) shows a narrow hydroxyl band at 3449 cm^{-1} compared to raw chitosan. The bands observed at 2881 and 1481 cm^{-1} are assigned to asymmetric C-H stretching and asymmetric CH_3 deformation, respectively.



Scheme 1: Formation of TMC carbonate from chitosan.

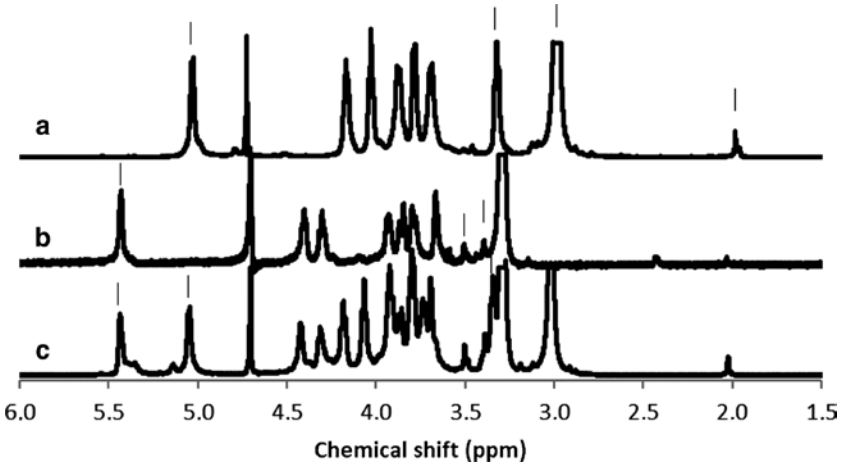


Fig. 1: ^{1}H NMR spectra of chitosan derivatives in D_2O (a) protonated DMC; (b) TMC with DQ of 95 %; and (c) protonated TMC with DQ of 48 %.

Fig. 2: IR spectra of chitosan derivatives (a) chitosan; (b) DMC; and (c) TMC with DQ of 95 %.

Formation and characterization of TMC

The *N*,*N*-dimethylation of chitosan increases the nucleophilicity of nitrogen atoms and facilitates the exhaustive *N*-methylation in milder reactional conditions. Table 1 presents the quaternization assays of DMC. Utilization of a strong base during the reaction of quaternization favors the *O*-methylation due to partial deprotonation of hydroxyl groups and leads to an undesired decreasing of solubility in water. Therefore, selective *N*-methylation of DMC was firstly realized with iodomethane using sodium bicarbonate as a weak base in a solvent mixture containing an amount of water (i.e. 10 % v/v). TMC synthesized with sodium bicarbonate in methanol or DMF/H_2O (runs 1–3) was obtained in the protonated form demonstrating that this base is not sufficiently strong enough to deprotonate the -$NH(CH_3)_2^+$ group in this protic solvent system, which is consistent with the pK_a values. Quaternization (run 3) using bicarbonate was achieved with a DQ of 48 % without *O*-methylation. The DQ was limited by the protonation of amine groups. ^{1}H NMR spectrum of protonated TMC synthesized with sodium bicarbonate (runs 1–3) reveals two methyl peaks at 3.3 and 3.0 ppm attributed to TMU and DMU, respectively. In addition, the IR spectrum of TMC shows a strong band at 1480 cm^{-1} corresponding to asymmetric CH_3 deformation, that is characteristic of highly *N*-methylated chitosan salts including *N*,*N*,*N*-trimethylammonium salts.[11] TMC (run 3) is soluble in water and insoluble in methanol, ethanol, acetone, and diethyl ether.

Table 1: The quaternization assays of DMC in various conditions.

Run	Solvent[a]	Base	Time	Methylation (%)				DA[b]
				TMU	DMU	O-3	O-6	
1	MeOH	$NaHCO_3$	8 d	24	75	nd[c]	nd	3.8
2	H_2O/DMF	$NaHCO_3$	4 h	33	72	nd	nd	2.5
3	H_2O/DMF	$NaHCO_3$	8 h	48	55	nd	nd	2.5
4	MeOH/H_2O	NaOH	47 h	82	25	68	42	1.2
5	MeOH/H_2O	NaOH	72 h	89	2	48	23	1.3
6	MeOH/H_2O	Na_2CO_3	22 h	70	14	6	6	3.4
7	MeOH/H_2O	Na_2CO_3	46 h	95	0	8	9	2.6
8	H_2O/DMF	Na_2CO_3	46 h	55	41	nd	nd	2.0
9	H_2O/DMF	Na_2CO_3	24 h	59	43	nd	nd	2.3

[a]In case of solvent mixtures, the proportion was 10:90 v/v; [b]degree of acetylation; [c]not detectable.

To circumvent the protonation of amine groups during the reaction, sodium bicarbonate was replaced by sodium hydroxide, a stronger base. Utilization of this base in a water/DMF mixture allows reaching more complete and selective *N*-alkylation. However, hydroxide ion favoured the *O*-methylation reaction and the hydrolysis of amide groups (runs 4, 5). The literature reported the incorporation of 50 % of water in the solvent mixture to prevent this secondary reaction was reported.[11] Moreover, tetramethylammonium iodide was obtained as by-product due to *N*-methylation of dimethylamine, which was produced by the DMF decomposition in alkali medium. The tetramethylammonium iodide, eliminated by ultrafiltration, shows strong IR bands at 3012, 1483, 1403, 1396 and 944 cm^{-1} that could be superimposed with infrared band of TMC. The IR spectrum of TMC synthesized in presence of aqueous sodium hydroxide shows a stronger band at 1483 cm^{-1} compared to the reaction with sodium bicarbonate due to higher DQ. Moreover, a weak IR band is observed at 1658 cm^{-1} and is attributable to the carbonyl stretching of *N*-acetylglucopyranosyl units. TMC iodide (DQ of 46 %) is soluble in water and insoluble in methanol, ethanol, acetone and diethyl ether.

To avoid decomposition of DMF and to use a green solvent, the quaternization of DMC was realized in a water-methanol mixture with iodomethane and sodium hydroxide (run 4) leading to a DQ of 82 % but also to 6-*O* and 3-*O*-methylation with a DS of 42 % and 68 %, observed respectively at 3.43 and 3.35 ppm in ^{1}H NMR. This assay shows that sodium hydroxide favours *O*-methylation even when the reaction was performed in protic medium (runs 4–5).

In the objective to circumvent the reaction of *O*-methylation, TMC was synthesized with weaker bases such as sodium bicarbonate and sodium carbonate in an aqueous solution. Bicarbonate anion was firstly studied to avoid ionic crosslinking from divalent anions that will decrease the solubility of TMC and could therefore decrease the rate of quaternization reaction. On the other hand, the formation of TMC using carbonate anion lead a water insoluble product and to a heterogeneous reaction, which had the advantage to greatly facilitate the purification of TMC as carbonate salt. Anion in TMC carbonate can be replaced by addition of an acid solution such as aqueous hydrochloric acid. A DQ up to 95 % (runs 6, 7) was reached after 46 h with low *O*-methylation (17 %). The DQ was calculated from integration of the peak at 3.3 ppm compared to pyranosyl peaks. No peak at 3.0 and 5.0 ppm is detectable, so the degree of *N*,*N*-dimethylated units is 0 %. In addition, only 2.6 % of units are acetylated suggesting that DQ is higher than 95 %, taken into account the *N*-acetylglucosamine units. The quaternization can be therefore considered as complete and quantitative. The reaction was also attempted in H_2O/DMF using sodium carbonate (runs 8–9), the DQ was below 60 % and no *O*-methylation was observed.

Characterization of TMC and quantification of the DQ

Pyranosyl peaks of TMC (DQ of 95 %, run 7) in ^{1}H NMR spectrum are well separated and are composed of seven peaks corresponding to an integration of one hydrogen atom (Fig. 1). In the case of TMC with a DQ

of 48 %, NMR peaks of pyranosyl hydrogen atoms of *N*,*N*-dimethylated units (DMU) and *N*,*N*,*N*-trimethylated units (TMU) are both observed and well resolved. As shown in Figs. 1 and 3, the quaternization influence not only the chemical shift of methyl groups (3.0–3.3 ppm), but also the chemical shift of all pyranosyl hydrogen atoms, especially H-1 that moved from 5.0 to 5.4 ppm for quaternized units. These chemical shifts are not affected by the level of quaternization of the TMC. Smaller peaks in the anomeric region at 5.14 and 5.35 ppm of TMC with DQ of 48 % are also observed that could came from the neighboring of di- and trimethylated units.

TMC with a degree of acetylation of 20.1 % was also synthesized using reactional conditions in run 7. When the DQ of this TMC, in the non-protonated form, was determined from the integration of the methyl peak at 3.3 ppm compared to pyranosyl integration, a total degree of nitrogen atom substitution of 125 % was obtained. This total degree of substitution was attained from the addition of degrees of acetylated units (24.9 %), DMU (11.1 %) and TMU (89 %). This degree of substitution over 100 % is illogical and proves that this method of quantification of the DQ has some bias that could be attributable to the superposition of H-2 signal with the $(CH_3)_3N$ peak. The NMR spectrum of DMC (Fig. 1) shows that H-2 of DMU is partly overlapped, even with 600 MHz NMR spectrometer, downfield to the methyl peak of TMU at 3.3 ppm in the protonated form, which is currently used for quantification of the DQ. With protonated TMC, the quantification using the $(CH_3)_3N$ integration achieved to a DQ of 66.8 % and a degree of DMU of 13.1 %, leading a total degree of nitrogen atom substitution of 100 %. This quantification method applied to the non-protonated TMC clearly overestimates the DQ value.

When the DMU are protonated, the integrations of the two anomeric peaks (δ 5.0 and 5.4 ppm) at room temperature (rt) can also be used to quantify the DQ of TMC obtained from DMC. Those peaks possess the advantage to be free of other pyranosyl signals and easy to distinguish. The H-1 peak of non-protonated DMU was overlapped with the water signal at rt. In literature, some authors have performed their analyses at 80 °C to overcome this difficulty [20]. If the TMC sample was not completely dissolved, the higher solubility of TMC in deuterated water with DQ during sample preparation could also result to an overestimation of the DQ by NMR spectroscopy. In our work, the protonation of DMU avoids these complications. The quantification method involving integration of anomeric hydrogen peaks is easier to apply and is more accurate due to the subtraction of integration errors coming from the superposition of H-2 and trimethylammonium peaks that is accentuated using lower field NMR spectrometers (i.e. 300–400 MHz). The quantification method using anomeric peaks is more accurate for higher DQ values due to a higher signal-to-noise ratio. H-1 NMR chemical shifts of chito-oligomers and *N*-acetyl chito-oligomers are 5.4 and 5.2 ppm [22], respectively, explaining that this latest quantification method is limited to the analysis of TMC obtained from DMC.

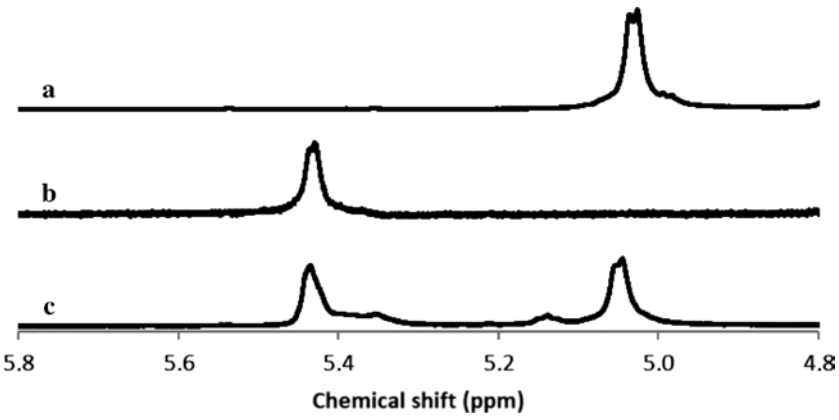


Fig. 3: ^1H NMR spectra of chitosan derivatives in D_2O of the H-1 signal (a) protonated DMC; (b) TMC with DQ of 95 %; and (c) protonated TMC with DQ of 48 %.

Conclusions

Our work shows that water-soluble TMC chloride with DQ up to 95 % and low *O*-methylation can be synthesized by selective *N*-methylation of DMC, prepared by Eschweiler-Clarke reaction, with iodomethane in methanol/water mixture. This quaternization process is a simple and almost organic solvent free methodology where the TMC carbonate can be easily recovered. ^{1}H NMR spectra of these highly quaternized TMC have showed that integration of H-1 peaks can be useful for the determination of DQ. This method is simple and possesses the advantage to imply a clear spectral window. Furthermore, this quaternization procedure paves the way to further selective transformations on chitosan derivatives and to improve biomedical properties of chitosan derivatives.

Acknowledgements: We thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for its financial support.

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